Sample Preparation of Human Serum Combined with Cleavable ICAT® Reagents to Enhance Low Level Protein Analysis

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INTRODUCTION

There has been an increased interest in the use of isotope coded affinity tags for the simultaneous quantification and identification of proteins in a complex mixture. However, a major difficulty in analyzing complex samples, such as human serum, is the dynamic range of protein concentrations present in the sample. For example, human serum albumin (HSA) constitutes 57-71% of total serum protein and γ-immunoglobulin (IgG) ranges from 8-26%. Removal of these two proteins alone clears about 75% of the total protein present in serum, therefore allowing detection of the remaining lower abundant proteins present. Combining serum depletion with the Cleavable ICAT® Reagents for assay, which further reduces sample complexity, increases the number of sequences that can be identified in a single MS/MS experiment and dynamic range. Here we describe the use of Poros® anti-HSA and Protein G depletion cartridges combined with the Cleavable ICAT Reagent technology and LC-MS techniques to identify lower abundant proteins in human serum.

MATERIALS AND METHODS

Removal of albumin: 3 μL of AB human serum (Heat inactivated; Pel-Freez Clinical Systems, LLC®) was diluted 1:10 with phosphate buffered saline, pH 7.2 (PBS) and passed through a 200 μL Poros anti-HSA cartridge (0.5 mL/min), the flowthrough (FT) collected . The column was further washed with 500 μL of PBS and the combined (FT) and subsequent elute (EL) were collected. The FT was subjected to further depletion of γ-immunoglobulin (IgG).

Removal of γ-immunoglobulin: IgG was removed by placing the anti-HSA combined FT sample onto a 200 μL Poros Protein G cartridge equilibrated with PBS. The column was further washed with 500 μL of PBS and again, the combined FT was collected. Elution of bound proteins was performed using 1 mL of 12 mM HCL. After collection, samples were dried in a vacuum concentrator prior to subsequent SDS-PAGE or labeling techniques.

Labeling samples with cleavable ICAT reagents: Separate samples were labeled with either the heavy or light Cleavable ICAT Reagent individually reduced with TCEP, then alkylated with cleavable ICAT Reagents and subjected to tryptic digestion at 37°C overnight. according to the protocols supplied in the Cleavable ICAT® Reagent Kit for Protein Labeling (Applied Biosystems).

Isolation and Analysis of ICAT reagent labeled peptides: Samples were cleaned up using a cation exchange cartridge and cysteine-containing peptides were isolated by the ICAT Reagent Cartridge-Avidin followed by TFA treatment to remove the linker containing the biotin moiety as described in the Cleavable ICAT Reagent Kit for Protein Labeling (Applied Biosystems).

One Dimensional SDS Polyacrylamide Gel Electrophoresis (1-D SDS-PAGE): Samples subjected to SDS-PAGE were done using a Novex® 10-20% Tricine gel in a Novex XCell mini gel electrophoresis system (Invitogen™ life technologies). Protein bands were visualized by staining with SimplyBlue™ SafeStain (Invitogen life technologies) followed by destaining with water.

Mass Spectrometry Analysis The ICAT reagent-labeled peptides were analyzed by LC-MALDI MS/MS using the LC Packings Ultimate LC / ProBot robotic MALDI spotter and AB 4700 Proteomics Analyzer with GPS Explorer™ software v2.0 with automated database searching (using Mascot Search Engine). The LC run was performed with a 45 minute 5% to 40% acetonitrile/0.1% TFA gradient with 20 second fraction collection onto the MALDI target.

RESULTS

Figure 1. One Dimensional SDS-PAGE of human serum fractionation

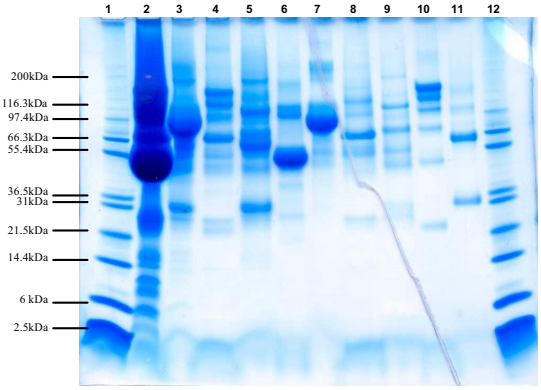


Figure 1: Electrophoresis of the serum depletion fractions before (even) and after (odd) ICAT labeling. Lanes 1 & 12) Mark 12 Protein Standard; Lanes 2 & 3) non-depleted serum ; Lanes 4 & 5) HSA depleted serum; Lanes 6 & 7) anti-HSA eluted fraction ; Lanes 8 & 9) HSA & IgG depleted serum; Lanes 10 & 11) Protein G elute

Table 1. Top 30 proteins identified from crude human serum (no removal of IgG or albumin – gel lane 3)

Protein Summary			
Rank	Protein Name	ICAT Peptides
1	Human Serum Albumin In A Complex With Myristic Acid And Tri-Iodobenzoic Acid	44
2	(V00494) reading frame HSA [Homo sapiens]	43
3	(NM_001063) transferrin precursor [Homo sapiens]	20
4	(X99549) gamma 3 immunoglobulin constant heavy chain [Homo sapiens]	7
5	(AK058037) unnamed protein product [Homo sapiens]	7
6	Ig G1 H Nie [Homo sapiens]	5
7	(AF027159) immunoglobulin gamma heavy chain [Homo sapiens]	7
8	(AJ250170) immunoglobulin heavy chain constant region [Homo sapiens]	6
9	Chain A, Human Transferrin N-Lobe Mutant H249q	7
10	Ig heavy chain V region precursor - human	5
11	(AJ010442) immunoglobulin kappa light chain [Homo sapiens]	5
12	Ig kappa chain C region (allotype Inv(1,2)) - human (fragment)	4
13	(NM_000186) H factor 1 (complement); H factor-1 (complement) [Homo sapiens]	5
14	(K00422) haptoglobin precursor [Homo sapiens]	4
15	protein Len,Bence-Jones [Homo sapiens]	4
16	X-Ray Crystal Structure Of Human Ceruloplasmin At 3.0 Angstroms	2
17	(BC014367) Similar to orosomucoid 1 [Homo sapiens]	2
18	(AF184765) IgG2 heavy chain [Homo sapiens]	3
19	(AC004682) haptoglobin-related protein precursor [Homo sapiens]	2
20	(J03048) hemopexin precursor [Homo sapiens]	4
21	(U88581) transferrin [Homo sapiens]	3
22	protein Rei,Bence-Jones [Homo sapiens]	2
23	(AF414429) alpha-1-B glycoprotein precursor [Homo sapiens]	2
24	(AB027444) anti HBs antibody light-chain Fab fragment [Homo sapiens]	2
25	(NM_000014) alpha 2 macroglobulin precursor [Homo sapiens]	1
26	(AF283666) SNC66 protein [Homo sapiens]	2
27	(D67012) alpha2-HS glycoprotein [Homo sapiens]	3
28	Chain A, Crystal Structure Of Human Beta-2-Glycoprotein-I (Apolipoprotein-H)	2
29	complement factor H precursor, short splice form - human	2
30	Immunoglobulin J chain	2

The majority of proteins identified belonged to the protein groups of albumins, transferrins and immunoglobulins.

Table 2 . Top proteins identified after removal of human serum albumin on an anti-HSA affinity cartridge – gel lane 5

Protein Summary			
Rank	Protein Name	ICAT Peptides
1	(NM_001063) transferrin precursor [Homo sapiens]	9
2	(M12525) transferrin [Homo sapiens]	6
3	(M12523) alloalbumin Venezia [Homo sapiens]	4
4	(AK058037) unnamed protein product [Homo sapiens]	2
5	(AF027159) immunoglobulin gamma heavy chain [Homo sapiens]	2
6	(NM_005143) haptoglobin [Homo sapiens]	1
7	(AF272774) factor VII active site mutant immunoconjugate [Homo sapiens]	2
8	(U07989) immunoglobulin kappa light chain [Homo sapiens]	2
9	(U88581) transferrin [Homo sapiens]	2
10	(AJ390260) immunoglobulin heavy chain [Homo sapiens]	3
11	(NM_000064) complement component 3 precursor [Homo sapiens]	2
12	Chain H, Immunoglobulin G1	2
13	(AF283666) SNC66 protein [Homo sapiens]	2
14	Chain I, P14-Fluorescein-N135q-S380c-Antithrombin-Iii	2
15	protein Len,Bence-Jones [Homo sapiens]	2
16	Chain A, Crystal Structure Of Human Beta-2-Glycoprotein-I (Apolipoprotein-H)	1
17	(X05006) S-protein [Homo sapiens]	1
18	JC-kappa protein - human	2
19	Zinc-alpha-2-glycoprotein precursor (Zn-alpha-2-glycoprotein) (Zn-alpha-2-GP)	2
20	(NM_000186) H factor 1 (complement); H factor-1 (complement) [Homo sapiens]	2

Proteins remaining after the albumin depletion include transferrin, with the majority of proteins belonging to the immunoglobulin classes such as light and heavy chains of both kappa and gamma immunoglobulins. Some other serum proteins were also observed such as alpha-glycoproteins.

Table 3 . anti-HSA affinity cartridge analysis – gel lane 7

Protein Summary			
Rank	Protein Name	ICAT Peptides
1	(NM_000477) albumin precursor; PRO0883 protein [Homo sapiens]	30
2	(V00495) serum albumin [Homo sapiens]	28

Albumin was found to be the only major protein which bound to (and eluted from) the anti-HSA cartridge – demonstrating the high specificity of these cartridges for albumin.

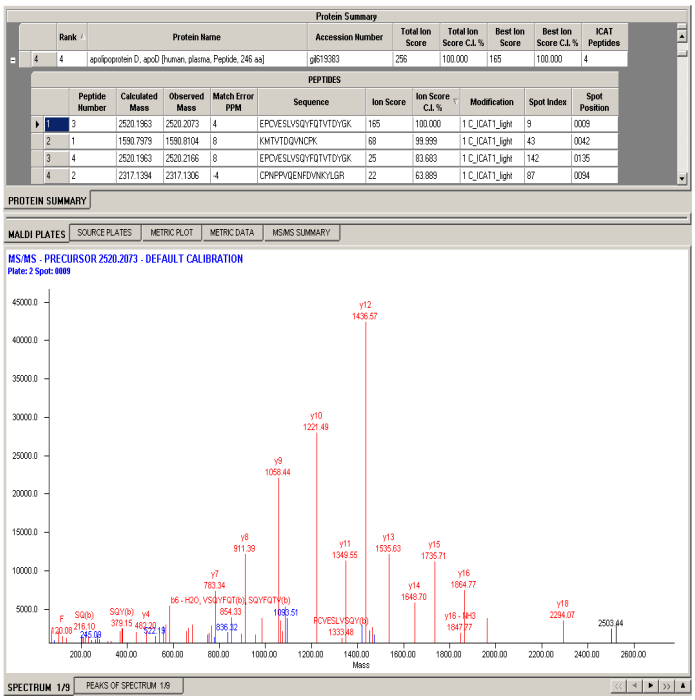
Table 4. Top proteins identified from serum depleted of both albumin and IgG – gel lane 9

	Rank	Protein Name	ICAT Peptides
1	1	(NM_001063) transferrin precursor [Homo sapiens]	17
2	2	(NM_000477) albumin precursor; PRO0883 protein [Homo sapiens]	8
3	3	haptoglobin Hp2 [Homo sapiens]	8
4	4	apolipoprotein D, apoD [human, plasma, Peptide, 246 aa]	4
5	5	Chain A, Human Transferrin N-Lobe Mutant H249q	4
6	6	(NM_000506) coagulation factor II precursor; prothrombin [Homo sapie	5
7	7	(D67013) alpha2-HS glycoprotein [Homo sapiens]	3
8	8	Ig alpha-2 chain - human (fragment)	5
9	9	Ig kappa chain C region - human	2
10	10	(AL389978) Immunoglobulin heavy chain variant [Homo sapiens]	5
11	11	(BC016369) Unknown (protein for MGC:27165) [Homo sapiens]	5
12	12	(AJ309319) anti-peptide/MHC complex HLA-A1/MAGE-A1 monoclonal	3
13	13	(U88581) transferrin [Homo sapiens]	4
14	14	(AL049744) dJ177P10.1.1 (H factor 1 (complement) isoform 1) [Homo s	2
15	15	(NM_000607) orosomucoid 1 precursor; Orosomucoid-1 (alpha-1-acid g	2
16	16	Chain B, Antithrombin Iii	3
17	17	(NM_144646) immunoglobulin J polypeptide, linker protein for immunogl	2
18	18	Alpha-1B-glycoprotein	2
19	19	vitamin D-binding protein - human	2
20	20	Chain A, Crystal Structure Of Human Beta-2-Glycoprotein-I (Apolipoprot	5
21	21	protein Tro alpha1 H,myeloma [Homo sapiens]	4
22	22	protein Len,Bence-Jones [Homo sapiens]	2
23	23	(NM_000014) alpha 2 macroglobulin precursor [Homo sapiens]	2
24	24	(NM_000062) complement component 1 inhibitor precursor; serine (or c	1
25	25	X-Ray Crystal Structure Of Human Ceruloplasmin At 3.0 Angstroms	1
26	26	(M11465) alpha-1-antitrypsin [Homo sapiens]	2
27	27	(BC002963) Unknown (protein for MGC:1652) [Homo sapiens]	2
28	28	(NM_000412) histidine-rich glycoprotein precursor; histidine-proline rich	3
29	29	(NM_001710) complement factor B preproprotein; B-factor, properdin; C	3
30	30	alpha-2-glycoprotein (Zn) - human	1

This table shows those proteins of highest confidence following depletion of albumin and γ-immunoglobulins not previously identified from the crude sample (Table 4, examples of MSMS in Figures 2, 3 & 4) and not classed as albumin or IgG. Using the ICAT strategy to simplify the mixture (Cys-peptides), increases the distribution of proteins identified in the mixture. This initial study was limited to a minimal sample amount with no further post-labeling fractionation steps prior to LC-MALDI MS/MS which may have allowed a greater depth of protein ID.

Figure 2. Apolipoprotein D identification from depleted human serum sample

a. Confident Identification of Apolipoprotein D Using GPS Explorer™ Software showing protein identification, protein groups as well as the peptides which matched this protein.



b. MSMS Spectrum of 2520.2073 m/z from 4700 Proteomics Analyzer showing correlation with GPS Explorer™ software data (shown in a)

Table 5. Analysis of Protein G bound fraction – gel lane 11

	Rank	Protein Name	ICAT Peptides
1	3	(Y14735) immunoglobulin kappa heavy chain [Homo sapiens]	12
2	5	(BC019046) Similar to immunoglobulin heavy constant gamma 3 (G3m marker) [Homo sapiens]	12
3	7	(BC019337) Similar to immunoglobulin heavy constant gamma 3 (G3m marker) [Homo sapiens]	12
4	1	(M12523) alloalbumin Venezia [Homo sapiens]	11
5	8	(BC016381) Similar to immunoglobulin heavy constant gamma 3 (G3m marker) [Homo sapiens]	11
6	2	(AJ390264) immunoglobulin heavy chain [Homo sapiens]	10
7	6	(AK058037) unnamed protein product [Homo sapiens]	10
8	4	Chain A, Fab Fragment Of Engineered Human Monoclonal Antibody A5b7	7
9	9	(AB027436) anti TNF-alpha antibody light-chain Fab fragment [Homo sapiens]	7
10	12	(J00221) immunoglobulin gamma-4 heavy chain [Homo sapiens]	7
11	11	protein Len,Bence-Jones [Homo sapiens]	6
12	13	(AJ250170) immunoglobulin heavy chain constant region [Homo sapiens]	6
13	15	Chain H, Immunoglobulin G1 (Igg1) (Mcg) With A Hinge Deletion	6
14	10	protein Rei,Bence-Jones [Homo sapiens]	5
15	14	(AB022650) anti-Entamoeba histolytica immunoglobulin gamma heavy chain [Homo sapiens]	5

The Protein G cartridge was shown to be specific for the γ-immunoglobulins. This is illustrated in the table above with the majority of proteins identified in this elution fraction being γ-immunoglobulins

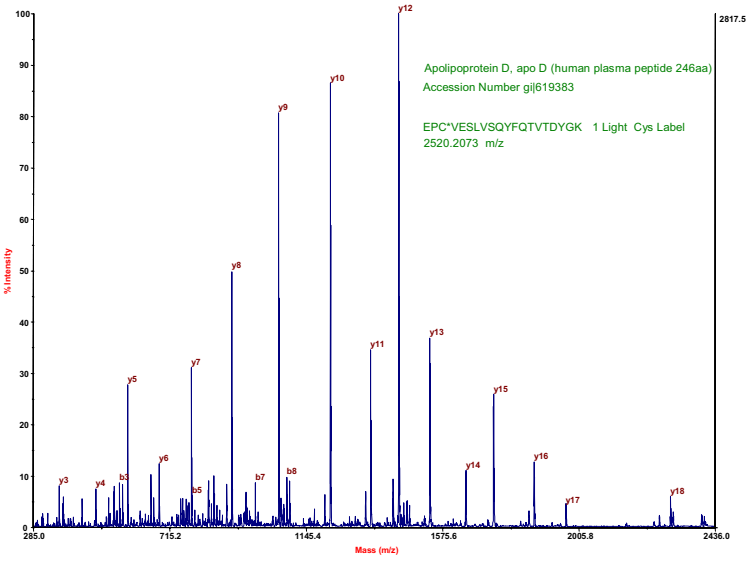
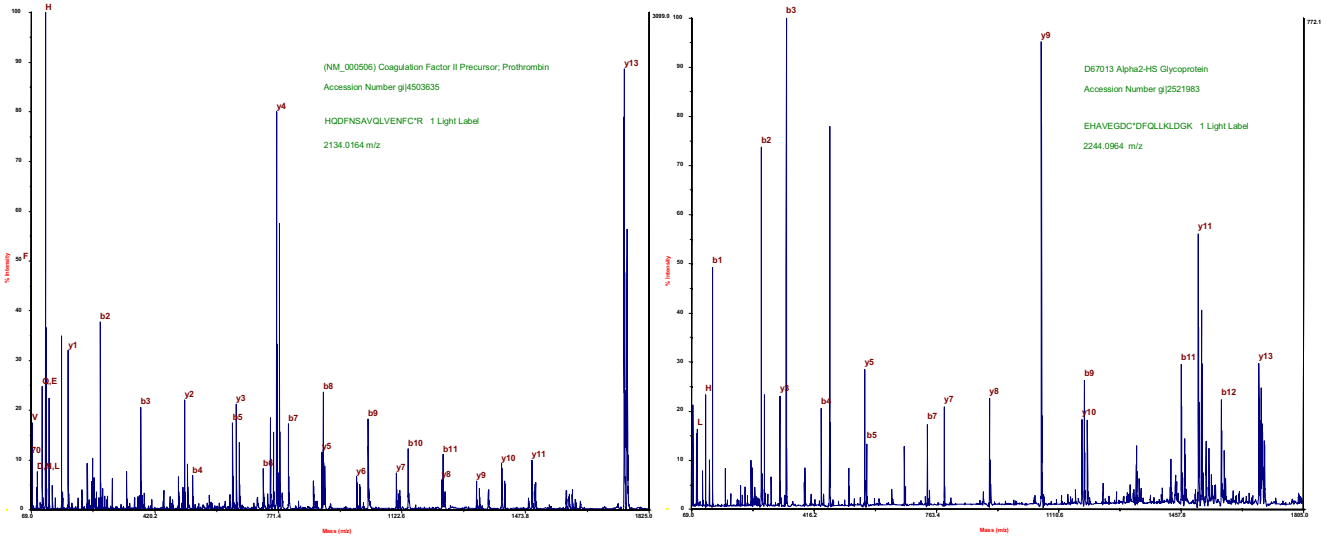


Figure 3. Prothrombin identification from depleted human serum sample

Figure 4. Alpha2-HS Glycoprotein identification from depleted human serum sample



CONCLUSIONS

•With no depletion of abundant proteins (Table 1), the majority of proteins identified were albumins, transferrins and immunoglobulins.

•The anti-HSA and Protein G cartridges are specific for albumin and γ -immunoglobulins, respectively.

– Tables 3 and 5 show the bound proteins identified were albumin and of the immunoglobulin family. Other proteins identified were of low confidence.

•The final depleted serum sample contains additional proteins, identified with high confidence (Best Ion Score C.I. >90%), otherwise not seen in the crude serum sample such as apolipoprotein D, prothrombin and alpha2-HS glycoprotein.

•Further mining of serum samples will require either

– LC fractionation or the use of 1D SDS-PAGE/cleavable ICAT reagents[1] by separating other more abundant proteins such as transferrin and macro- & hapto- globulins from the less abundant proteins or
– Increasing the scale of labeling; as this study was performed using only 100 μg starting material.

REFERENCES

1) Yuen, S., *et. al.*; ASMS Poster #492; Investigation of a Mammalian Cellular Model for Differential Protein Expression Analysis using 1-D PAGE and Cleavable ICAT® Reagents

TRADEMARKS/LICENSING

Applied Biosystems